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Abstract: Familial hypercholesterolemia (FH) is an autosomal dominant disease which results in 2-3-fold elevated cholesterol levels and in accelerated atherosclerosis. FH is caused by small mutations or larger rearrangements in the low-density lipoprotein receptor (LDLR). Here, we report that screening the LDLR gene in a Swiss family (n = 15) with clinical symptoms of FH by combined single strand conformation polymorphism and long-distance PCR identified a novel 1.3 kb deletion in the LDLR. The deletion eliminated exon 4 of the LDLR presumably by recombination between two identical 25 bp repeats present in intron 3 and 4. The 25 bp sequence in intron 3 is part of an Alu repeat, whereas no homology to Alu repeats was found for the intron 4 region. This 1.3 kb LDLR deletion allele cosegregated with elevated cholesterol levels over three generations. Even on high-dose statin therapy, carriers of the deletion averaged 1.6 times higher cholesterol levels and 1.9 times higher apolipoprotein B-100 (apoB-100) levels than non-carriers who had lipid and apoB-100 levels within the range of the Swiss population. Most affected members of the first and second generation of this family had experienced a first myocardial infarction (MI) before the age of 55 years and most LDLR gene deletion carriers older than 40 years showed severe coronary artery disease (CAD). Hence, we conclude that deletion of exon 4 in the LDLR gene drastically decreases low-density lipoprotein binding leading to severe hypercholesterolemia.

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Detection of a Novel Exon 4 Low-Density Lipoprotein Receptor Gene Deletion in a Swiss Family with Severe Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is an autosomal dominant disease which results in 2-3-fold elevated cholesterol levels and in accelerated atherosclerosis. FH is caused by small mutations or larger rearrangements in the low-density lipoprotein receptor (LDLR). Here, we report that screening the *LDLR* gene in a Swiss family (n = 15) with clinical symptoms of FH by combined single strand conformation polymorphism and long-distance PCR identified a novel 1.3 kb deletion in the LDLR. The deletion eliminated exon 4 of the LDLR presumably by recombination between two identical 25 bp repeats present in intron 3 and 4. The 25 bp sequence in intron 3 is part of an Alu repeat, whereas no homology to Alu repeats was found for the intron 4 region. This 1.3 kb *LDLR* deletion allele cosegregated with elevated cholesterol levels over three generations. Even on high-dose statin therapy, carriers of the deletion averaged 1.6 times higher cholesterol levels and 1.9 times higher apolipoprotein B-100 (apoB-100) levels than non-carriers who had lipid and apoB-100 levels within the range of the Swiss population. Most affected members of the first and second generation of this family had experienced a first myocardial infarction (MI) before the age of 55 years and most *LDLR* gene deletion carriers older than 40 years showed severe coronary artery disease (CAD). Hence, we conclude that deletion of exon 4 in the *LDLR* gene drastically decreases low-density lipoprotein binding leading to severe hypercholesterolemia.
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Key words: Familial hypercholesterolemia; Exon 4 deletion; Low-density lipoprotein receptor; Long-distance PCR.

Abbreviations: apoB-100, apolipoprotein B-100; CAD, coronary artery disease; FH, familial hypercholesterolemia; LDLR, low-density lipoprotein receptor; MI, myocardial infarction.

Introduction

Cardiovascular disease accounts for the majority of morbidity and mortality in the western countries. Most forms of cardiovascular disease involve atherosclerotic vascular changes in the coronary, cerebral, renal, and peripheral circulation leading to angina pectoris, myocardial infarction (MI), stroke, renal failure, and claudication. Hypercholesterolemia is a well recognized risk factor for atherosclerosis of central and peripheral arteries (1). In the western countries, one of the most common genetic disorders which results in elevated cholesterol levels is familial hypercholesterolemia (FH). This autosomal dominant disorder with a prevalence of about 1/500 in the western countries is caused by mutations in the (*LDLR*) gene (2). The LDLR defect impairs the catabolism of LDL and results in elevation of plasma LDL-cholesterol (2). Untreated heterozygous FH patients have 2–3 times elevated cholesterol levels and have a 100-fold increased risk to die from coronary artery disease (CAD) before the age of 40 years, while a 5-fold increased risk is still observed for patients over 40 years (3).

Over 770 different mutations in the *LDLR* gene have been reported (http://www.ucl.ac.uk/fh) which are more or less equally spread over the entire gene. The majority of mutations in the *LDLR* gene are nucleotide substitutions or small deletions and insertions; however, about 12% of all entries in the LDLR database are deletions or insertions larger than 1 kb. Two hundred and sixty-nine *LDLR* mutations (Germany: 95, France: 82, Italy: 69 and Austria: 20) are known in Central Europe (http://www.ucl.ac.uk/fh). In Switzerland, only 2 mutations are known so far: V408M and P664L (4).

We decided to screen for the LDLR in a family with clinical manifestation of FH by single strand conformation polymorphism (SSCP) to detect small mutations, and by long-distance PCR (5) to detect larger rearrangements (6). Using this screening strategy we detected a 1333 bp deletion of exon 4 in the *LDLR* gene, ranging from intron 3 to intron 4, which cosegregated with elevated cholesterol levels in a Swiss family with severe hypercholesterolemia.

Materials and Methods

Fifteen members of a family with clinical manifestations of FH were enrolled from the outpatient clinic of the University Hospital in Zurich. There was a family history of premature cardiovascular disease with elevated cholesterol levels. Patients underwent a medical history- and physical examination. Standard 12-lead electrocardiography and exercise testing were done if appropriate. Blood samples were taken for blood lipid profile and genetic testing for *LDLR* gene defects. The study was approved by the local Ethics Commitee and all patients included gave informed consent to participate.

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DNA was extracted with a Qiamp® DNA Blood Mini Kit (QIAGEN, Venlo, The Netherlands). Long-distance PCR (Expand™ long template PCR System: Roche, Basel, Switzerland) was performed as previously described (5) with minor modifications on a GeneAmp® PCR System 9600 (Applied Biosystems, Foster City, USA): The $MgCl_2$ concentration were 2.75 mM for amplification of fragments 1, 4 and 5, and 2.25 mM for fragments 2 and 3. An annealing temperature of 62 °C for fragments 1, 2 and 4, and 58 °C for fragments 3 and 5 was used. Elongation times were 13 min for fragments 2, 4 and 5, 17 min for fragment 1, and 11 min for fragment 3. The total number of cycles was 30. The first 15 cycles were realized by the parameters defined above followed by 15 cycles with increased elongation time (15 s per cycle). PCR products were analyzed on a 0.8% agarose gel. Running conditions were 100 V during 6 h followed by ethidium bromide staining and ultraviolet (UV) detection.

To define the exact breakpoints of the deletion, we amplified the region between intron 1 (primer 4 (P4)) and 5 (P2), intron 2 (P5) and 5 (P2), intron 3 (P6) and 5 (P2), and between exon 4 (P7) and intron 5 (P2) (for primers see Table 1). Fifty µl PCR reactions included 400 nanomolar (nM) of the upper and lower primer, 500 micromolar (µM) 2′-deoxynucleotide 5′-triphosphate (dNTP's), 2.5 units Expand™ long template enzyme mixture in 1× Expand™ long template buffer 3, and approximately 400 ng genomic DNA. The DNA was amplified with an initial denaturation step of 94 °C for 2 min, followed by 30 cycles at 94 °C for 10 s, 60 °C for 30 s, and 68 °C for 8 min, followed by a final elongation step at 68 °C for 7 min. PCR products were analyzed on a 0.8% agarose gel.

For routine detection of the exon 4 deletion, a 2.4 kb fragment was amplified using an upper primer (P1) in intron 3 and a lower primer in intron 5 (P2). The intron 3 to intron 5 region was amplified in a 50 µl reaction containing 400 nM solution of each primer, 500 µM each dNTP, 2.5 units of Expand™ long template Enzyme Mix in buffer 3 of the Expand™ long template PCR system. The PCR was performed under the following conditions: an initial denaturation step of 2 min at 94 °C, followed by 15 cycles for 10 s at 94 °C, 30 s at 60 °C, and 4 min at 68 °C. Another 15 cycles were performed with the same parameters, except that in every elongation step the incubation time was increased by 15 s.

To define the deletion breakpoints, the PCR fragment from the wild-type and the mutant allele (PCR with primers P1 and P2) was subcloned with an Original TA Cloning® Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The plasmid isolation was performed with a QIAprep Spin Miniprep Kit (QIAGEN, Venlo, The Netherlands). Plasmid inserts were then sequenced on an ABI Prism 310 (Applied Biosystems, Foster City, USA), with the chemistry and running conditions recommended by the manufacturer. Downstream sequencing primer was P1, upstream sequencing primer was P3.

Plasma total cholesterol and triglyceride values were determined on a Hitachi 747 (Roche, Basel, Switzerland), high-density lipoprotein (HDL)-cholesterol and apolipoprotein B-100 (apoB-100) values were measured on a Cobas Integra 700 (Roche, Basel, Switzerland) with standard methods supplied by the manufacturer.

Results

Initially, the deletion in the LDLR was detected by two independent long-distance PCR amplifications in four family members. First, in the amplified fragment encompassing the promotor region to exon 5 of the *LDLR* gene and second, in the PCR product encompassing the genomic region from exon 2 to exon 10 (data not shown). Since both amplifications detected the deletion, the deletion breakpoint must be located between exon 2 and exon 5 of the *LDLR* gene.

To scan for the exact breakpoints of the deletion, PCR products of different length were amplified on the genomic DNA from a FH sibling (Figure 1). The PCR products were designed to span from introns 1 to 5, 2 to 5, 3 to 5, and from exon 4 to intron 5, dissecting the region into four smaller stretches. Amplification of introns 1–5 and 2–5 produced two products representing the wild-type and the deletion allele, whereas amplification of intron 3 to intron 5 and amplification of exon 4 to intron 5 resulted in only one signal. Hence, the 5′deletion breakpoint is possibly located in intron 3, and due to the approximate size of the deletion (1.3 kb) the 3′ deletion breakpoint should be located in intron 4.

To determine the exact breakpoints of the deletion, we subcloned the amplified wild-type and deletion allele from an affected sibling into pCR®2.1. We amplified the region between intron 3 and intron 5, which resulted in a wild-type product of 2399 bp and in a product of 1066 bp harboring the deletion. Sequencing of the two subclones revealed that the shorter allele lacked 1333 bp deleting the entire coding region for exon 4. At the deletion breakpoints, two identical 25 bp direct repeats with the sequence TTTTTGAGATGGAGTCTCACTCTGT were present (Figure 2) suggest-

Table 1 Oligonucleotides used in this study.

Primer	5′ position*	Sequence**	3′ position*
P1	75540	CCTACCTACTTTCTGGAATAAATCTG	75515
P2	73143	CGCCCTCTGGCTTCACAAATC	73163
P3	73784	TGGTGAAACCCCATCTCTACTAAATAG	73810
P4	79759	ATAGACACAGGAAACGTGGTCA	79738
P5	77294	CAGTGGGTCTTTCCTTTGAGTGACA	77270
P6	74752	AATGGGCTGGTGTTGGGAGACT	74731
P7	74499	AACGACCCCGACTGCGAAGA	74480

*Positions are given according to the *Homo sapiens* chromosome 19 clone CTB-164O19 (GenBank Accession No. AC011485) wherein the entire *LDLR* gene is present. **Sequences are shown 5′ to 3′.

ing that the deletion originated from illegitimate recombination.

To investigate if this exon 4 deletion in the LDLR was responsible for hypercholesterolemia, we genotyped 15 family members for the exon 4 deletion and investigated cosegregation of the deletion with hypercholesterolemia. To simplify genotyping, we established a PCR which amplifies the region from intron 3 (849 bp upstream of exon 4) to intron 5 (80 bp downstream of exon 5) encompassing the exon 4 deletion. Amplification of the wild-type allele produced a PCR product of 2.4 kb, whereas amplification of the allele with the exon 4 deletion produced a 1.1 kb product (Figure 3).

Over three generations, all carriers of the exon 4 deletion, had markedly elevated total cholesterol, LDL-cholesterol and apoB-100 levels despite cholesterol-lowering therapy (mean: 8.2 mmol/l, 6.2 mmol/l and 1.7 g/l, n = 10), and only slightly elevated triglyceride levels (mean: 1.8 mmol/l, n = 10) (Table 2). Two previously not treated carriers of the third generation had mean total cholesterol, LDL-cholesterol, apoB-100, and triglyceride levels of 10.2 mmol/l, 8.5 mmol/l, 2.2 g/l, and 1.5 mmol/l, respectively (n = 2). In contrast, the non-affected siblings had plasma lipid and apoB-100 levels in the range of the Swiss population (mean: 5.0 mmol/l, 3.0 mmol/l, 0.9 g/l, and 1.0 mmol/l, respectively, n = 3). It is noteworthy that even on high-dose cholesterol-lowering therapy (simvastatin 80 mg/d, atorvastatin 80 mg/d), carriers of the exon 4 deletion had elevated cholesterol levels and most of the carriers needed combined lipid-lowering therapy and LDL-apheresis. Moreover, three affected members of this family had experienced a premature MI (patients no. 4, 5, and 6) and two more affected members (no. 1 and 9, Figure 3) showed CAD as demonstrated by angiography. These two subsequently underwent percutaneous coronary intervention and coronary artery bypass grafting.

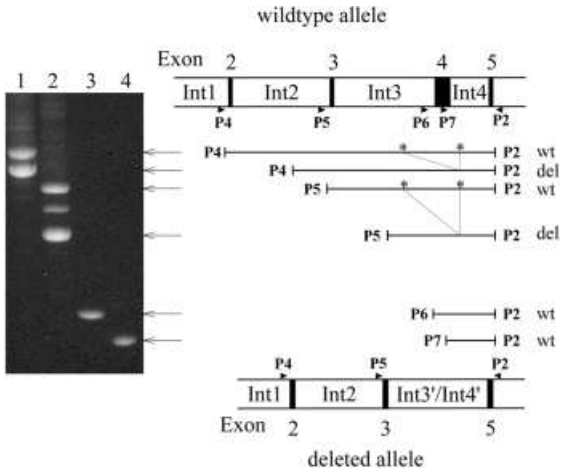

Figure 1 Approximate localization of the deletion breakpoints. The genomic region from intron 1–5 (lane 1), intron 2–5 (lane 2), intron 3–5 (lane 3), and exon 4 to intron 5 (lane 4) was amplified and separated on an agarose gel (left side). A schematic illustration of the experiment is shown on the right side. These results indicate that the deletion breakpoints must be localized in intron 3 and 4. Two signals appear at the amplification of the genomic sequence from intron 2–5, representing the wild-type allele (upper signal) and the allele that carries the 1.3 kb deletion (lower signal). In lane 2, where the genomic sequence from intron 3–5 was amplified, three signals are visible, whereas the signal in the middle supposedly derives from unspecific amplification. The upper and lower signals represent the wild-type (wt) and the deleted (del) allele, respectively.

Discussion

This study describes a novel exon 4 deletion in the LDLR, which cosegregates over three generations with

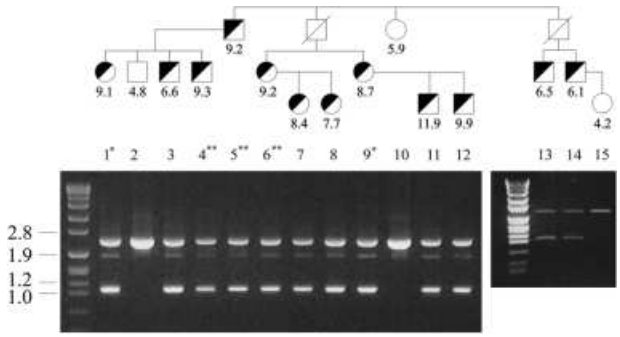

Figure 3 Genetic analysis of the Swiss family with a 1.3 kb deletion. Top: Pedigree of the affected Swiss FH-patients. Half-filled symbols indicate affected-, blank symbols denote non-affected members of the family, whereas squares represent males and circles females. The family member marked with a slash has deceased after three cardiac infarctions. Cholesterol levels at presentation are indicated in mmol/l below the symbols. Patient numbering corresponds with Table 2. Patients with severe CAD*, or MI**, respectively. Bottom: An ethidium bromide-stained agarose gel is shown, prepared after genetic testing of the exon 4 deletion. The signal, which migrates as a 2.4 kb fragment represents the PCR product of the wild-type allele, and the signal at 1.1 kb represents the PCR-product of the mutant allele.

```
INTRON 3  5'ACTACCTTTTTTAAAATTTCTTTCTTTTTTGAGATGGAGTCTCACTCTGTTTGCCCAGGCTGGAGTTCAGTGGTGCAATC 3'
          **************************************************        *     *  * *      ***
DEL EX 4  5'ACTACCTTTTTTAAAATTTCTTTCTTTTTTGAGATGGAGTCTCACTCTGTGATTTTTTTTCATCTCTAAATTTCCTACATC 3'
            *    ****     *** ***  ************************************************************
INTRON 4  5'TTTTTTCTTTTCTTTCTTTATTTTGTTTTTGAGATGGAGTCTCACTCTGTGATTTTTTTCATCTCTAAATTTCCTACATC 3'
```

Figure 2 Schematic illustration of the exon 4 deletion breakpoint area. At the breakpoints of the deletion, two identical 25 bp direct repeats are present. The upper and lower sequence stretches show wild-type sequences from intron 3 and 4, respectively, whereas the sequence in the middle represents the deleted allele. Asterisks indicate identical bases.

Table 2 Patient data.

Patient no.	Sex	Age	Drug treatment	Long PCR	Chol. total (mmol/l)	HDL (mmol/l)	Chol/ HDL	LDL (mmol/l)	Tg (mmol/l)	ApoB-100 (g/l)
1	F	40	+	del	9.1	1.5	6.1	6.9	1.6	1.8
2	M	39	–	wt	4.8	1.5	3.3	3.0	0.9	0.9
3	M	38	+	del	6.6	1.5	4.4	4.8	0.8	1.2
4	M	36	+	del	9.3	1.1	8.4	6.0	4.9	1.9
5	M	74	+	del	9.2	1.6	5.8	6.5	2.5	1.9
6	F	47	+	del	9.2	0.9	10.8	7.4	2.2	2.2
7	F	26	–	del	8.4	0.7	11.7	6.8	1.9	2.0
8	F	29	+	del	7.7	1.2	6.4	5.8	1.6	1.7
9	F	58	+	del	8.7	1.0	8.7	7.0	1.6	1.9
10	F	84	–	wt	5.9	1.7	3.6	3.7	1.3	1.0
11	M	39	–	del	11.9	1.3	9.5	10.1	1.1	2.4
12	M	36	+	del	9.9	0.9	11.5	8.3	1.7	2.1
13	M	51	+	del	6.5	1.6	4.1	4.7	0.6	1.4
14	M	48	+	del	6.1	1.7	3.6	4.2	0.5	1.2
15	F	25	–	wt	4.2	1.6	2.7	2.3	0.7	0.8
∅	del untreated		(n=2)		10.2	1.0	10.6	8.5	1.5	2.2
∅	del treated		(n=10)		8.2	1.3	7.0	6.2	1.8	1.7
∅	wt		(n=3)		5.0	1.6	3.2	3.0	1.0	0.9

Clinical data of investigated FH family members: Values are actual concentrations of the respective parameters. The patient-numbering corresponds with Figure 3. Column 4 shows whether drug treatment was realized (+) or not (–). Chol = cholesterol, Tg = triglyceride. The last three rows resume averaged values of 3 wild-type (wt)-, 10 affected and treated (del treated)- and 2 affected but not treated (del untreated) family members.

elevated cholesterol levels and with clinical manifestation of FH. Even on high-dose statin therapy, mean cholesterol levels in affected members of the family still averaged 1.6 times the level of the non-affected siblings (8.2 mmol/l, n = 10 *vs.* 5.0 mmol/l, n = 3).

Although the penetration of the exon 4 allele is high and all carriers showed elevated cholesterol levels, there are still interindividual differences in cholesterol levels. For example, out of three siblings on high-dose statin therapy who are all heterozygous for the exon 4 deletion, two showed clearly elevated cholesterol levels (patient no. 1: 9.1 mmol/l and patient no. 4: 9.3 mmol/l total cholesterol; Table 2), whereas the third sibling had only slightly elevated cholesterol levels (patient no. 3: 6.6 mmol/l). Intriguingly, all three siblings had identical apolipoprotein E genotype (ε2/3) and none had the familial defective apoB-100 mutation (data not shown), both known factors to influence cholesterol levels (7, 8). As was previously observed, there are other genetic and environmental factors that affect cholesterol levels in FH patients (9–11). Kajinami *et al.* (12) reported that plasma cholesterol levels in some heterozygotes with an exon 2–3 deletion was essentially normal, although the receptor affinity for LDL-particles was reduced to 40% in homozygotic carriers. Vega *et al.* (9) reported that such an inconsistent penetration of the FH mutation could originate from changes in LDL input rates and LDL catabolic rates. Although cholesterol levels varied between carriers of the exon 4 deletion in the Swiss family, no carrier showed normal cholesterol levels, suggesting that the penetration of the exon 4 deletion is high and that the exon 4 deletion results in a receptor-deficient phenotype.

Exon 4 codes for three of the seven cystein-rich ligand-binding repeats of the LDLR and plays an essential role in receptor binding activity. In the *LDLR* gene, exons 2–6 code for the seven ligand-binding domains, whereas exon 4 alone codes for repeats number 3–5 which are in the center of the ligand-binding domain (13). Transcription from the exon 4 deletion allele is predicted to lead to an mRNA lacking exon 4, considering that the splicosome will use the splicing donor site in intron 3 and use the next available acceptor site in intron 4. Translation of this truncated mRNA is predicted not to prematurely terminate through a frameshift or stop mutation but rather to translate a LDLR lacking the 127 amino acids of exon 4. This truncated LDLR missing three ligand-binding repeats is predicted to have reduced or absent binding capacity since it has been shown that deletion of the ligand-binding domains results in decreased binding capacity for apoB-100 (12, 14, 15). For example, in a similar mutation where exon 5 is deleted which codes for one cysteine-rich ligand-binding repeat, the receptor lost its function to bind apoB-100 containing LDL (14). Furthermore, Esser *et al.* (16) analyzed LDLRs with several truncated ligand-binding domains in transfected COS-M6 cells for binding to LDL. They found that consecutive deletion of ligand-binding repeats correlated with decreased receptor-binding to LDL. When the receptor lacked binding repeats 1–3, LDL-binding was reduced to 31% of the wild-type receptor. However, when binding repeats 1–5 were absent, ligand-binding was totally

abolished (16). From these data we conclude that the exon 4 deletion receptor, which lacks the ligand-binding repeats 3, 4, and 5, has markedly reduced or absent ligand-binding activity. As mentioned before, this is supported by clinical data from the Swiss family with hypercholesterolemia where the exon 4 deletion cosegregates with highly elevated cholesterol levels. Intriguingly, this is not the case for all LDLR mutations (17).

The exon 4 deletion in the *LDLR* gene is flanked by two 25 bp direct repeats suggesting that illegitimate recombination between the two repeats caused the deletion. Two other deletions in the LDLR were reported to originate from recombination, both lacking exon 5: FH-Aarhus-3 (18) and FH-Paris-1 (= FH626) (14) which is also described as FH London-2(19). Investigation of the deletion breakpoints in these alleles revealed that repeats belonging to the Alu families were present on each side of the deletion (14,18). Such a mechanism may have created the deletion of exon 4, since at the deletion breakpoints two 25 bp direct repeats are present which are part of several Alu subfamilies (20). The region of the deletion breakpoint in intron 3 contains a sequence stretch that shows homology with Alu repeat subfamilies Sc, Sg, Sx, and Y wherein the direct repeat is present. The region in intron 4 lacks homology with Alu repeats, however, the 25 bp direct repeat is identical to intron 3, suggesting that this short repeat triggered recombination and deletion of exon 4 in the LDLR.

In summary, this study shows an exon 4 deletion in the LDLR that cosegregates with hypercholesterolemia and clinical symptoms of FH. The severe phenotype of this deletion indicates that lack of the three ligand-binding domains encoded by exon 4 results in a receptor-deficient phenotype.

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